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Chemoenzymatic synthesis of glycoproteins

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The majority of the world's best-selling biotherapeutics are glycoproteins. However their production using cellular expression systems invariably produces inseparable mixtures of materials which differ in their attached carbohydrates. As in many cases correct carbohydrate structure is vital for *in vivo* efficacy, the development of methods for the production of glycoproteins in homogeneous form has become a significant scientific objective. Here a brief overview of recent progress in the production of homogeneous glycoproteins, including monoclonal antibodies, will be discussed, centring on the use of endo-β-*N*-acetylglucosaminidase (ENGase) enzymes for protein glycoengineering.

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Glycoproteins: issues and therapeutic applications

Protein glycosylation is the most diverse form of post-translational modification, and crucially affects important protein properties [1]. Many proteins of clinical and therapeutic significance are glycosylated, most prominently immunoglobulin G (IgG) antibodies. Recombinant monoclonal antibodies (mAbs) are without doubt the most significant new class of therapeutic agent to be developed in recent times. Numerous studies have demonstrated that Fc-receptor-mediated effector functions of IgGs are highly dependent on the structure of attached carbohydrates (glycans); correct glycosylation is therefore important for the therapeutic efficacy of some mAbs [2].

The glycan structures of other recombinant glycoprotein therapeutics may also be vital for their *in vivo* efficacy. For example, recombinant versions of human lysosomal enzymes, developed to treat several of the lysosomal storage disorders (LSDs), are *N*-glycosylated, and their trafficking to the lysosome by the mannose-6-phosphate receptors (M6PR's) relies on phosphorylation of mannose residues of the protein's *N*-glycans. Proper glycosylation therefore plays an important role in the efficacy of some enzyme replacement therapies for LSDs [3].

The issue with the production of recombinant glycoproteins in mammalian (or plant or yeast) cell lines is the inherent complexity of the biosynthetic process, which inevitably results in a range of different glycan structures being attached to the same protein. This lack of control over product structure is exacerbated by the near indistinguishability of these different protein 'glycoforms,' (proteins possessing the same amino acid sequence but differing in their attached carbohydrates), making their separation before use impractical (if not impossible). The result is that all recombinant therapeutic glycoproteins, including mAbs, are currently manufactured and administered as mixtures of materials in which different oligosaccharide structures are linked to the same peptide chain. Indeed, the number of different glycoforms actually present in a clinically administered sample can be large; a total of more than 70 different glycans was identified as being present at Asn-297 during analysis of a group of 8 commercial therapeutic mAbs [4]. Access to pure single glycoforms of glycoproteins has therefore become a major scientific objective. Much work has been done on engineering the glycosylation machinery of different cell types (mainly yeast and plant) in order to try and address glycan structure and heterogeneity; unfortunately detailed discussion of these important studies is beyond the scope of this short review. Suffice to say that because of the 'secondary' glycosylation machinery present in these cases (i.e. numerous glycosidases and glycosyltransferases) protein expression even using highly engineered cells still generally produces mixtures of glycoforms, although these may be 'biased' towards particular glycoform subtypes. Amongst alternative approaches that have been adopted to access defined glycoproteins in completely homogeneous form, including the total chemical synthesis [5] of protein and oligosaccharide components, this short review will focus on recent developments in chemoenzymatic methods [6] for the remodelling of proteins that have themselves been produced by expression.

Glycoprotein remodelling: applications of ENGases (endo-β-*N*-acetylglucosaminidases)

The ENGases [7] are enzymes produced by a wide range of organisms that specifically cleave the diacetylchitobiose core [GlcNAcβ(1-4)GlcNAc] of N-linked glycans

between the two *N*-acetylglucosamine (GlcNAc) residues. Thus when they act on a glycoprotein the two products of hydrolysis are truncated *N*-glycan oligosaccharides, which are released, and a protein which possesses a single GlcNAc residue at any *N*-glycosylation sites (Figure 1). Their application as biocatalysts to achieve the reverse process, namely the attachment of *N*-glycans to GlcNAc residues, is the basis of a highly effective convergent method to access homogenous glycoproteins.

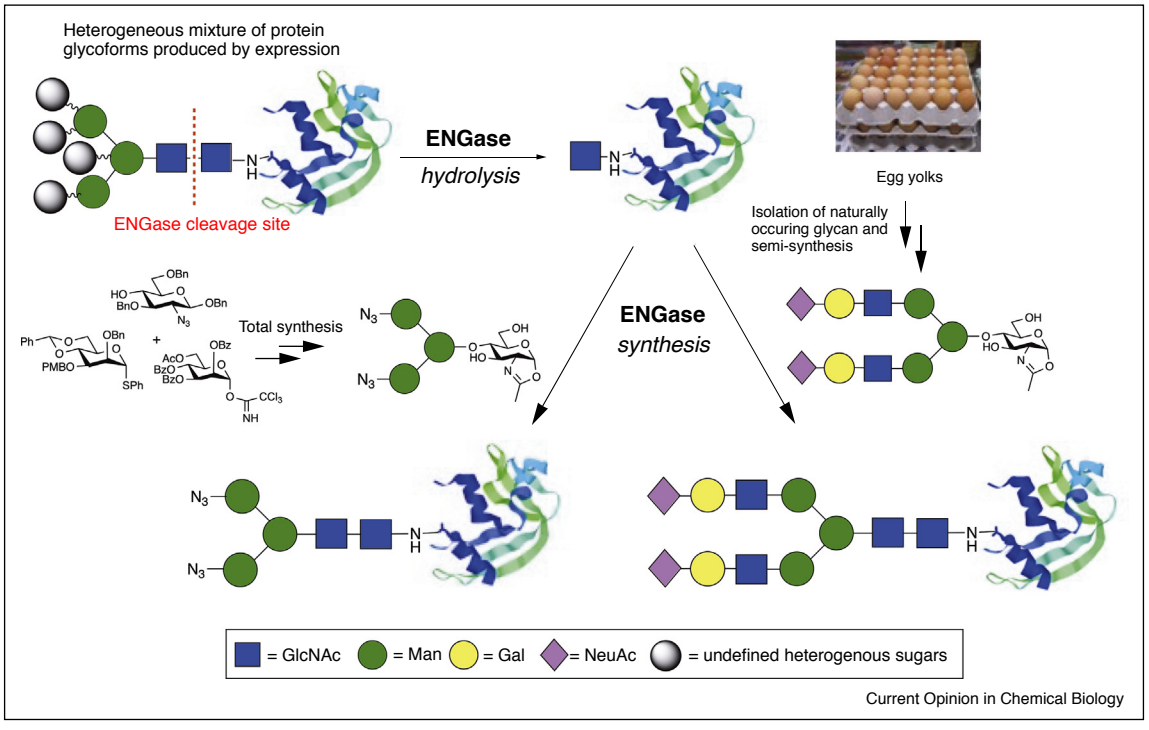
In vitro glycoprotein remodelling entails two steps (Figure 1) both of which are catalysed by an ENGase enzyme. Although it would appear paradoxical that the same enzyme could be used to achieve both hydrolysis and synthesis, two key advancements have made this process highly effective. The first was the demonstration by Fujita *et al.* [8] that *N*-glycan oxazolines could be used as activated donor substrates for ENGases. Oxazolines are high-energy intermediates on the ENGase-mediated hydrolytic pathway, and thus may be used to intercept GlcNAc residues in a synthetic process that is formally the reverse of hydrolysis. Moreover, and quite remarkably, they may now be readily accessed from the corresponding reducing sugar in a single step without the need for any protecting groups [9••], meaning that a wide variety of *N*-glycan structures may be readily accessed, either by chemical synthesis or isolation from natural sources [10].

The second key development was the production of mutant 'glycosynthase' ENGases by site-directed mutagenesis [11–13], Herein, judicious exchange of key active site residues produces mutant enzymes which cannot act hydrolytically, but which can still catalyse the synthetic attachment of *N*-glycans to GlcNAc residues using oxazolines as donors. Thus an irreversible and efficient synthetic process can be achieved, in which *N*-glycan structures can be attached to a protein that has a GlcNAc residue to act as the point of attachment.

The use of ENGases for the production of remodelled glycoproteins

RNase B has been the prototypical glycoprotein for remodelling using various ENGases and different *N*-glycan oxazolines. In the first step commercially available bovine RNase B is treated with a wild type (WT) ENGase (for example Endo H or Endo A) to cleave the heterogenous mixture of high mannose *N*-glycans at Asn-34 back to a single GlcNAc residue (the product is often termed

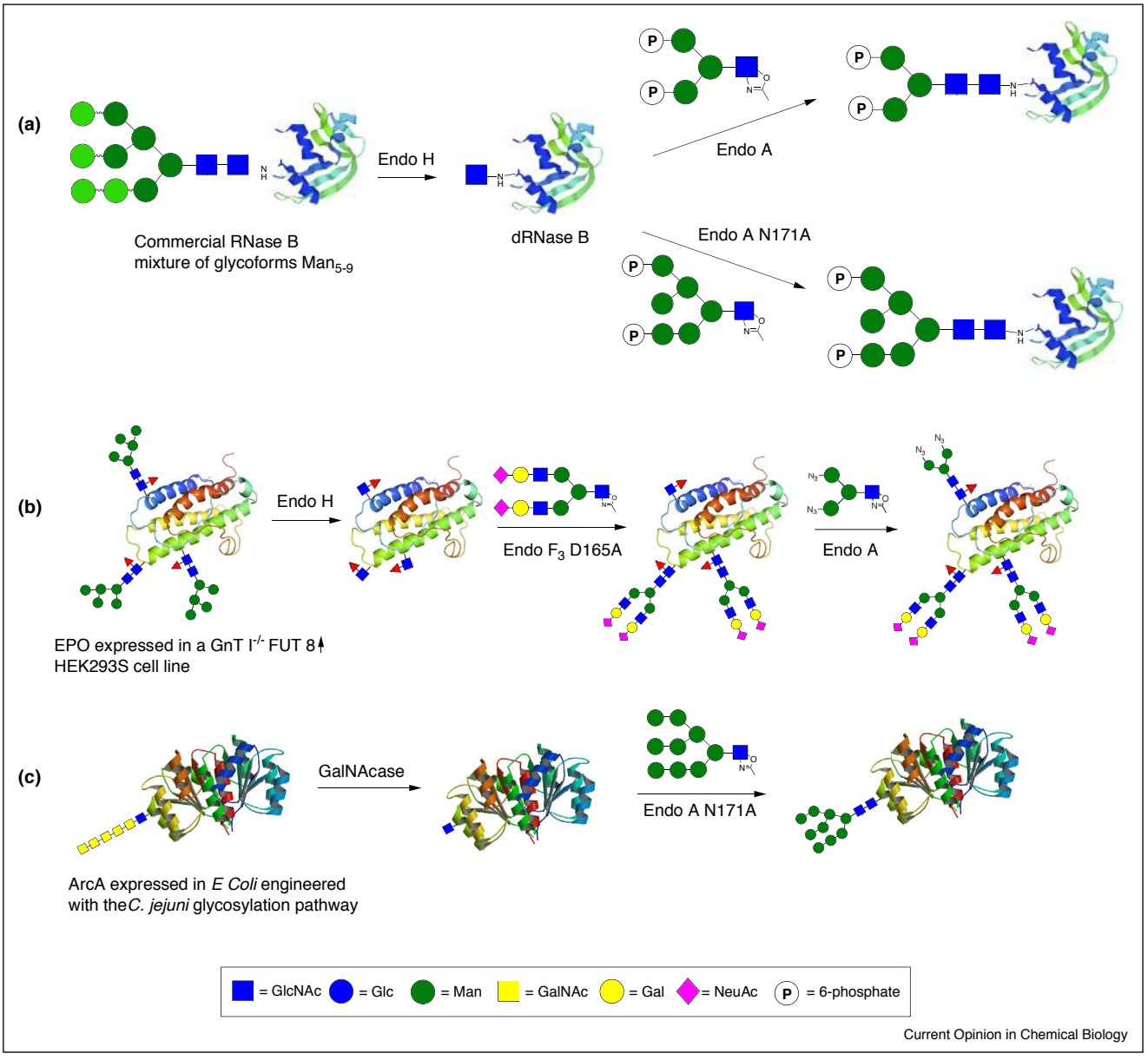
Figure 1



The use of ENGases to catalyse glycoprotein remodelling. In the first step the heterogenous mixture of glycoforms at the N-linked glycosylation site(s) produced during expression is trimmed back to a single GlcNAc residue by the action of an appropriate ENGase. In the second step an *N*-glycan oxazoline, either produced by total chemical synthesis or semi-synthesis using an *N*-glycan isolated from natural sources, is used for ENGase-catalysed glycosylation of these GlcNAc residues. The product is a remodelled glycoprotein bearing defined homogeneous *N*-glycans at designated Asn residues.

dRNase B). The resulting homogeneous protein can be used as a substrate for the ENGase catalysed attachment of a wide variety of *N*-glycans. Recently ENGase catalysis [14•,15•] has been applied to RNase B as a model protein to demonstrate that glycoengineering can produce proteins decorated in M6P-terminated glycans (Figure 2a). In these studies synthetic glycans comprising M6P residues were converted into oxazolines and transferred to dRNase B using both WT Endo A and Endo A mutants. Lysosomal protein trafficking is mediated by the mannose-6-phosphate receptors (M6PRs), which recognise *N*-glycans decorated with M6P residues. As mentioned previously the production of glycoproteins that possess optimal phosphorylation of mannose residues is therefore of particular significance for improving the efficacy of enzyme replacement therapies (ERTs) for the treatment of lysosomal storage disorders. This ENGase-mediated remodelling approach offers significant promise for ensuring complete phosphorylation of the glycans of recombinant ERT's.

Figure 2



Remodelling of glycoproteins using ENGases: **(a)** remodelling of commercial RNase B to produce homogenous glycoforms comprises mannose-6-phosphate terminated *N*-glycans; **(b)** remodelling of erythropoietin (EPO) expressed in a knockdown HEK293S cell line; **(c)** remodelling of ArcA expressed in an *E. coli* cell line engineered with the *C. jejuni* glycosylation pathway.

Erythropoietin (EPO) is another glycoprotein therapeutic for which *N*-glycans are essential for *in vivo* activity. Despite being produced by one of the landmark efforts in total synthesis [16], its large scale production will inevitably continue to be by expression. In a recent study [17•] a recombinant EPO was remodelled by ENGase catalysis. Initial studies met with failure—ENGases were not able to cleave the *N*-glycans (predominantly tetra-antennary complex) back to GlcNAc residues. Although this problem could be circumvented by expression in the presence of the mannosidase inhibitor kifunensine (an approach which produces only high mannose glycans), ENGases (e.g. an Endo M-N175A mutant) were then unable to attach glycans to non-fucosylated GlcNAc residues. A solution was found by expression in a HEK293 cell line, in which *N*-acetylglucosaminyltransferase I (GnT I) had been knocked out, and in which α-1-6-fucosyltransferase (FUT8, an enzyme which core fucosylates in the absence of GnT I) was overexpressed. Thus recombinant EPO was initially produced as mainly a $Man_5GlcNAc2Fuc$ glycoform at the three N-linked glycosylation sites. Cleavage back to a single fucosylated GlcNAc was now successful, and was followed by the attachment of sialylated complex bi-antennary glycans, using an ENGase glycosynthase mutant (Endo F3-D165A). Moreover site selectivity was also possible.

This work, although impressive and ultimately successful, does highlight some of the ongoing problems with the ENGase glycoengineering approach. Namely it is not possible to predict *a priori* which will be the best ENGase enzyme for the job, nor whether an ENGase will actually be able to act synthetically on a particular GlcNAc residue attached at a particular site on a particular protein. There are also some glycan structures which still may not be added synthetically, for example tetra-antennary (or higher) complex *N*-glycans.

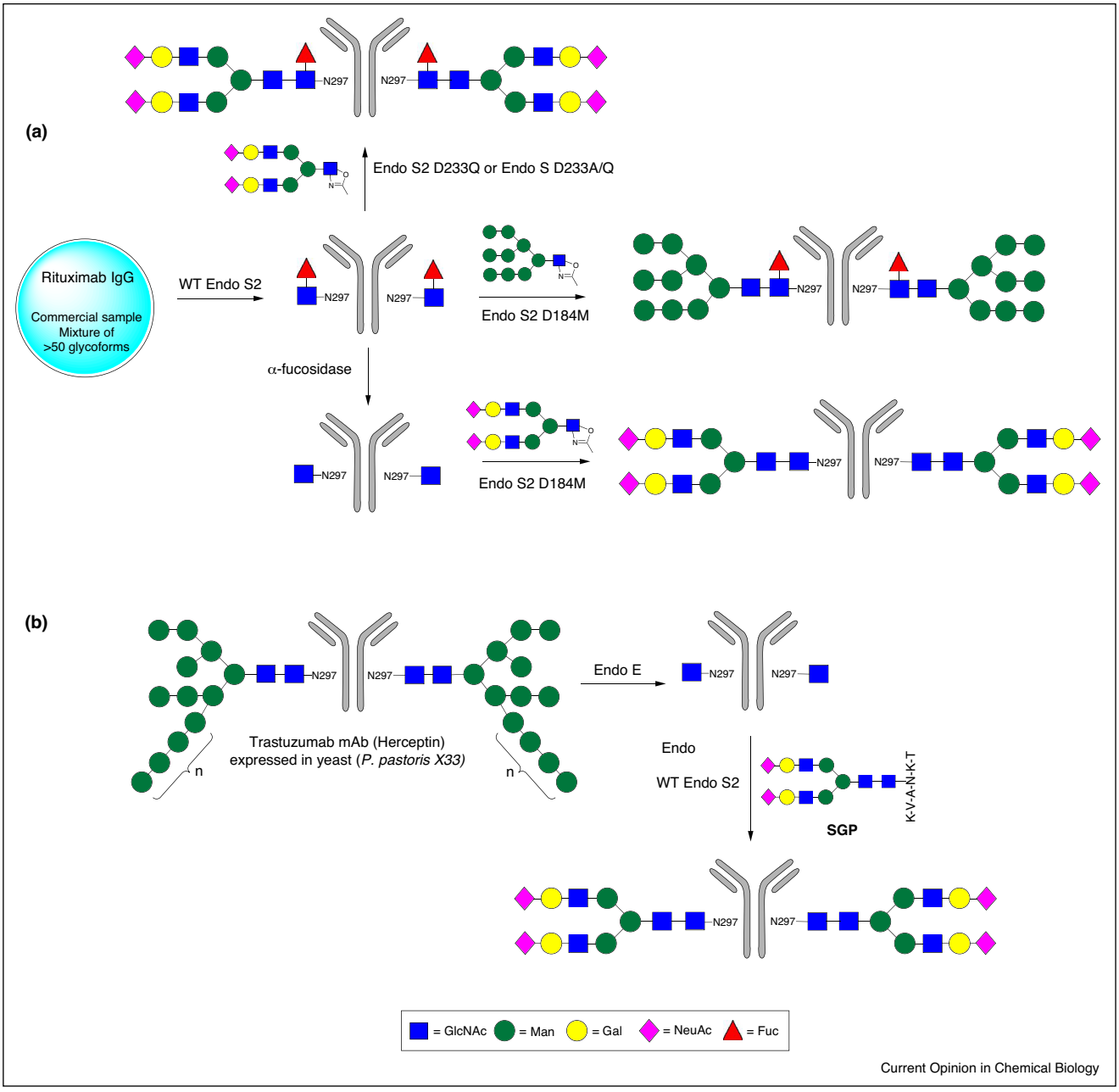
Glycoprotein substrates for ENGase-mediated remodelling may also be accessed by alternative mechanisms for asparagine glycosylation. Some prokaryotes, such as *Campylobacter jejuni*, are capable of N-linked protein glycosylation. However bacterial oligosaccharyltransferases (e.g. PglB from *C. jejuni*), which can glycosylate locally flexible regions in folded proteins [18], normally use donors containing rare non-mammalian sugars, and also require an extended acid consensus sequence (e.g. D/QXNXS/T as compared to the required mammalian sequon, NXS/T). Nonetheless extensive engineering of the *C. jejuni* glycosylation machinery and transfer into *Escherichia coli* has allowed the production of glycoproteins in *E. coli* (including a *C. jejuni* glycoprotein AcrA, and a single chain mammalian antibody) which possessed a poly-*N*-acetylgalactosaminylated-GlcNAc residue at N-linked sites [19]. Trimming with an exo-α-*N*-acetylgalactosaminidase produced glycoproteins bearing the requisite single GlcNAc residue at N-linked sites, and these underwent ENGase-mediated extension with oxazoline donors (Figure 2c). In a more recent approach trimming was completely avoided by the *in vitro* use of novel synthetic lipid linked donors [20] with recombinant *C. jejuni* PglB. The production and application of *N*-glycosyl transferases and their mutants from other bacterial sources (e.g. APGT from *Actinobacillus pleuropneumoniae*) also show promise for the production of appropriate substrates for ENGase extension [21].

The use of ENGases for the production of remodelled mAbs

The significant level of heterogeneity of glycan structures attached to mABs, together with the clinical and commercial importance of mAb-based therapies, has been a major driving force for application of the remodelling approach to IgGs. However at first studies only met with modest success, as many ENGases would not act on fully folded IgGs. A turning point came with the discovery that the family GH18 ENGase Endo S, an enzyme which preferentially acts on complex type *N*-glycans attached to folded IgG's, has useful synthetic activity for remodelling the glycans attached to the two Asn-297 residues of the Fc-regions of mAbs [22]. Recently the structural basis for Endo S's ability to act on complex *N*-glycans has been revealed [23]. The production of glycosynthase mutants of Endo S (D233A and D233Q) [24] allowed the first demonstration of efficient remodelling of therapeutic mAb (rituximab) with full-length complex biantennary structures, as well as asialylated complex and core structures (Figure 3a). This report stimulated further studies on remodelling of biotherapeutic mAbs such as Rituximab [25] and Herceptin [26•]. In the latter case an important, yet avoidable, side reaction was revealed; namely protein glycation when large excesses of oxazoline were used. More recent studies have added additional mutations to increase the synthetic utility of Endo S [27], whilst Endo S has also been used to remodel mAbs expressed in plant cell lines [28].

Following this surge of interest, glycosynthase mutants (D184M and D184Q) of a new enzyme called Endo S2 [29] have been developed by Li *et al.* [30••]. These ENGases have much wider applicability and are capable of transferring all three major types of *N*-glycan (high mannose, hybrid, and complex) to a range of IgGs, both with and without core fucose (Figure 3a). A key study on the remodelling of Rituximab clearly demonstrated the detrimental effect of core fucosylation on effector functions [31]. Other mutants of Endo S2 and their application for remodelling of rituximab have also been recently reported [32]. The glycation side reaction discovered during mAb remodelling with oxazolines [26•] has provoked a re-investigation of transglycosylation reactions using full length *N*-glycans as donors. Such reactions, the original synthetic application of ENGases before Shoda's demonstration of the superiority of oxazolines, are usually

Figure 3



Remodelling of IgGs using ENGases; **(a)** Endo S2 D184M and Endo S D233Q catalysed attachment of a glycans to remodel commercially produced rituximab; **(b)** remodelling of Herceptin (trastuzumab) expressed in yeast cells (*Pichia pastoris*) using WT Endo S2 to catalyse *trans*-glycosylation using a complex biantennary sialyl-glycopeptide (SGP) isolated from egg yolks.

much less efficient, requiring a very large excess of *N*-glycan donor and kinetic control as the reaction products are hydrolytic substrates for the ENGases.

Despite these obvious limitations some recent studies have been reported using the readily available donor sialyl glycopeptides: a complex biantennary N-linked glycopeptide, often simply called SGP which is available from egg yolks in hundred milligram quantities. For example, remodelling of Herceptin (trastuzumab) expressed in yeast cells (either a gene edited *Pichia pastoris* strain producing $Man_5GlcNAc_2$ glycans or an X33 strain producing polymannosylated glycans) was achieved using WT Endo S2-mediated transglycosylation with SGP (Figure 3b). De-glycosylation, either with Endo H or a new enzyme (Endo E) produced an IGg with a

single non-fucosylated GlcNAc at both N297 residues [33]. Although large excesses of Endo S and SGP were required the synthetic reaction was quite efficient, giving up to 80% of the remodelled antibody. Other recent studies have also revealed the potential use of other enzymes [34], or enzyme combinations for remodelling of mAbs, though in some cases just how this has been achieved is not entirely clear; for example, when two hydrolytically inactive mutants were used together with SGP as the donor [27].

Finally, very recently Giddens *et al*. have reported [35••] the site-selective remodelling of *N*-glycans of both the Fc (glycans attached to Asn-297 of the heavy chain) and Fab (glycans attached to Asn-88 of the heavy chain) of cetuximab, a therapeutic mAb used for treatment of a variety of cancers. This extremely elegant work hinged upon the complimentary selectivities of the Endo F3-D165A glycosynthase mutant, which only glycosylates core fucosylated GlcNAc residues, and the Endo S-D233A glycosynthase mutant, which only glycosylates GlcNAc residues at Asn-297 of IgGs. Key to the success of this site-selective remodelling was the development of a selective recombinant α-fucosidase derived from *Lactobacillus casei* (AlfC), which only removed core fucose from the GlcNAc residues at Asn-297.

Perspectives and conclusions

ENGase-mediated-remodelling of glycoproteins is an attractive approach that allows their production in homogenous form with a wide variety of glycans, the structure of which can be optimised for protein function. However some limitations still remain—notably with multi-antennary complex glycans [36], possible side reactions, and the difficulty of predicting *a priori* if an ENGase will be active synthetically on a particular GlcNAc of a particular protein. Endo S2 is a remarkably useful enzyme and has become the catalyst of choice for the glycoengineering of mAbs. Applications of ENGase remodelling for the production of mAbs and other therapeutic glycoproteins for clinical use appear to be just around the corner.

Conflict of interest statement

Nothing declared.

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- •• of outstanding interest

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